Original Article

Iran J Ped Hematol Oncol. 2017, Vol 7. No 2, 105-116

Promoter Methylation and Gene Expression in Human CD34$^+$ Stem Cells Derived Erythroid Lineage by MicroRNA

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Received: 24 June 2016 **Accepted:** 01 September 2016

Abstract

Background: Stem Cell differentiation is a process composed of vast variety of factors which are controlled by a network of certain mechanisms. This study aims to determine the possible role of DNA methylation, a potent regulator of VHL, ECAD and RUNX3 genes during Erythroid differentiation driven by miR-451.

Materials and Methods: To determine the methylation status of promoters and the expression levels of VHL, ECAD and RUNX3 genes, Methylation Specific PCR (MSP) and real-time PCR were used, respectively, on both Cord Blood CD34+ Hematopoietic Stem Cells and differentiated cells. To measure the expression levels of mir-451, mirna qpcr technique was used.

Results: Our findings demonstrated a similar methylation pattern for the target genes before and after differentiation by miR-451. However, the expression levels were significantly increased after differentiation. Gene expression and surface marker analysis results further confirmed the potential of miR-451 for driving erythroid differentiation from hematopoietic stem cells.

Discussion: Our findings ruled out DNA methylation effect on the regulation of VHL, ECAD, and RUNX3 genes during miR-451 mediated erythroid differentiation. However, having CpG islands in their promoters, these three genes are candidates to be controlled by methylation which may not able to be detected by MSP method.

Conclusion: Taken together in this study we have shown a successful erythroid differentiation mediated by miR-451 which is at least in part, independent of DNA methylation. Further understanding of the underlying mechanisms driven by eryhtroid differentiation may lead to therapeutic measures to alter disorders of hematopoietic stem cell differentiation.

Keywords: DNA methylation, Erythroid differentiation, Gene expression, MiR-451, Stem cell

Introduction

Myeloid, lymphoid, and erythroid lineages are three different cell lineages of hematopoietic system (1, 2). Differentiation into these three functional distinct lineages from hematopoietic stem cells (HSCs) includes myriad of molecular interactions that are able to regulate HSC self-renewal and lineage fate (1, 3-5). Identification of specific molecular pathways which regulate HSC differentiation, self-renewal, and proliferation remains a fundamental aim of either basic or clinical biology (5, 6). Proper lineage specification can be prevented by different genetic and epigenetic aberrations affecting differentiation and/or proliferation of HSCs and ultimately lead to severe diseases like myelodysplasia and leukemia

(7-9). Here, we evaluated three critical factors implicated in differentiation process in order to shed light into at least one of the possible mechanisms in this process. Von Hippel Lindau (VHL) is located on chromosome 3 [(3p25.3) (Exact Location; Chromosome 3, NC_000003.12 (10141635..10153670)] and codes for VHL protein(10) which degenerate the Hypoxia Inducible Factor (HIF) protein by ubiquitination(11). Runt-Related Transcription Factor 3 (RUNX3) is located on chromosome 1 [(1p36.11) (Exact Location; Chromosome 1, NC_000001.11 (24899511..24965158, complement)] that is also a cell cycle regulator and involved in differentiation(12). Moreover, RUNX3 performs as a tumor suppressor which is functionally disrupted in majority of malignancies (13-15). Previous studies show that down-regulation of RUNX3 is associated with DNA hyper methylation of RUNX3promoter (14). Calcium Dependent Adhesion-E (ECAD) is located on chromosome 16 [(16q22.1) (Exact Location; Chromosome 16, NC_000016.10 (68737290...68835542)] and is another critical factor implicated in differentiation process and has a significant role in intracellular adhesions which are required during tissue formation and differentiation (16). In addition to transcription factors, accumulating evidences have confirmed the regulatory role of micro-RNAs in the self-renewal and differentiation of stem cells (17-19) Based on previous studies, utilized miR-451 to differentiate the HSCs toward erythroid lineage (20-22). These findings suggested that promoter methylation of the above mentioned factors does not largely mediate their upregulation during miR-451 mediated erythroid differentiation or at least the changes are such tiny which is not detected by non-sensitive methods such as methylation specific PCR (MSP). Unraveling the sophisticated mechanisms controlling lineage commitment will enhance our perception on the etiology of diseases such as anemia, cancer, and some other hematological disorders.

Materials and Methods

Cell source

Umbilical cord blood was obtained following the vaginal and cesarean deliveries with informed consent by women to participate in this study at Sarem Hospital, Tehran, Iran. The blood was drained in sterile collection bags containing citrate phosphate dextrose as anticoagulant. To isolate the CD34+ cells, the bags were transported to cell culture laboratory in Tarbiat Modares University, Tehran, Iran.

Cell isolation

In order to primary isolation of mononuclear cells the cord blood was diluted 1:4 with Hydroxy Ethyl Starch and incubated for 45 minutes at room temperature. The mononuclear cells were isolated by layering the suspension over an equal volume of Ficoll-Hypaque 1.077 g/ml. After centrifugation for 30 min at 400g (1410 rpm), the cells at the interface were removed and washed three times with PBE (PBS/0.5% bovine serum albumin/5mM EDTA). To isolate CD34+ cells, the mononuclear cells were incubated with 500µl PBE and 50µl blocking antibody (Indirect CD34 MicroBead Kit, Milteny Biotech, Catalog no. 130-046-701) for 10 min at 2-8°C. Then, we added 50µl CD34-conjugated magnetic beads (Indirect CD34 MicroBead Kit, Milteny Biotech) and incubated for 45 min at 2-8°C. After the incubation time, 5ml of PBE was added to the suspension and centrifuged at 300g for 10 min at 4°C. Subsequently, the pellet was dissolved with PBS and mixed cautiously; then processed through a MACS magnetic separation column (Indirect CD34 MicroBead Kit). Cells conjugated to micro-beads then passed through the column in a magnetic field and CD34+ cells retained.

The column was then washed by PBE for three times.
Removing the magnetic field, target cells recovered into a 1.5ml Eppendorf tube by flushing the column with 1ml PBE.

CD34 expression analysis following the purification procedure

To confirm the purity of CD34+ hematopoietic stem cells, the surface marker (CD34) was analyzed using flow cytometry. The cells were incubated with phycoerythrin (PE) -conjugated murine monoclonal anti- CD34+ (Milteny Biotech) in PBS for 20 min in darkness at room temperature. Finally, the cells were washed three times and dissolved with PBS and analyzed by Partec PAS III flow cytometry device and the results were analyzed using Syflogic software.

Cell culture and expansion

To further expand the purified CD34+ cells derived from cord blood, the cells dispensed into 24 well plates and cultured at a density of 2×105 for seven days in incubator at 37℃ with 5% CO2. The medium used for cell culture and expansion was Stem Span (Stem cell Technologies, Vancouver, British Columbia, Canada) supplemented with recombinant human Flt3, TPO, and SCF with the concentration of 50ng/ml and 1% penicillin -streptomycin (Sigma, St Louis, MO, USA). Every 48 hours, half of the supernatant was replaced by a fresh medium. Harvesting, the cells were washed with PBS and divided into two parts. One part was used for subsequent culture for the differentiation purposes, and the second part was used for DNA and RNA extraction.

Induction of erythroid differentiation by miR-451

In order to differentiate the cells toward erythroid lineage the cells were cultured for seven days at a density of 1×105 cells per 400µl prepared IMDM medium supplemented with 20ng/ml SCF, 10% FBS, 100µl prepared Optimem Medium,1.25µl Lipofectamine 2000 and 2µl commercial miR-451 with the final concentration of 12.5 pM. The medium has been refreshed every 48 hours. To confirm the successful differentiation into erythroid lineage, we used both flow cytometric and Polymerase Chain Reaction (PCR) analysis on both CB-HSCs and cells after treatment with miR-451.

Assessment of differentiation efficiency

To further verify the differentiation, CD34 and CD71 as HSC and erythroid specific surface markers, respectively were analyzed using flow cytometry. Harvesting and washing the subjected cells with PBS, 100µl of cell suspension containing 1×106 cells were exposed to each Fluorescein isothiocyanate (FITC)-labeled monoclonal anti-CD34 and FITC-labled monoclonal anti-CD7. Beside, an isotype control-FITC was used to normalize the experiment. After incubation for one hour on ice in dark place, the cells were washed twice with PBS containing 2% bovine serum albumin (BSA) for flow cytometric analysis. A number of 1×105 cells were analyzed for the expression of CD34 and CD71 surface markers. To confirm the results, the mRNA levels of CD71 and CD235 were also analyzed using reverse transcriptase PCR. The products were resolved on 1.5% Tris-acetate-EDTA (TAE) agarose gel and Beta actin gene (ACTB) was used as an internal control of the experiment.

RNA extraction and Real-Time PCR

Total RNA extraction was performed using RNeasy Mini Kit (Qiagen) according to the manufacturer´s protocol. The quality of RNA was measured using spectrophotometry. Gel electrophoresis was also used to determine RNA integrity. CDNA was synthesized using 1st-Strand cDNA Synthesis Kit (Stratagene). Real-time PCR was carried out to evaluate the expression levels of VHL, ECAD and RUNX3 genes before and after

differentiation using ABI 7500 real-time PCR device. GAPDH housekeeping gene was used as an internal control for normalization of the results. Initially, 25 cycles of PCR amplification were performed then, 4µL was resolved on 1.5% Tris-acetate-EDTA (TAE) agarose gel to ensure the presence of subjected products. The length of product size for VHL, ECAD and RUNX3 genes were 123, 60, and 120bp, respectively. To perform the reaction, 12.5µl of 2X-SYBR Green PCR Master Mix (ABI), 1µl of each forward and reverse primer, 2µl of cDNA template, and 8.5 µl of RNase-Free water were added to a total volume of 25µl and the reaction were performed for 40 cycles of PCR. Acquired data were interpreted using Pfaffl calculations. The sequence of the primers in this step has been shown in Table I.

miRNA extraction and quantification

In order to evaluate the expression levels of miR-451 before and after differentiation, the total RNA was extracted from related cells using RNase Mini plus Kit (Qiagen). Afterward, total RNA was applied to synthesize cDNA using miRNA 1st-Strand cDNA Kit (Stratagene) according to the manufacturer's instructions. MiRNA expression was quantified using the miRNA QPCR kit (Stratagene) according to the manufacturers' protocol and the target expression level normalized to GAPDH expression. A control scramble sequence was applied to confirm the expression levels of miR-451. To perform the reaction, 12.5µl of 2X-SYBR Green PCR Master Mix (ABI), 1µl of forward and reverse primer, 2µl of cDNA template and 8.5 µl of RNase-Free Water were mixed to 25µl as total volume and the reactions were followed by 40 cycles of PCR. The sequence of the subjected primers in this step describes as below:

Forward: 5' GGAAGATCTTGACAAGGAGGACAG GAGAG 3'

Reverse: 5' CCCAAGCTTGCCTTGTTTGAGCTGG AGTC 3'

DNA extraction and bisulfite treatment

Genomic DNA extraction was performed using QIAamp DNA Mini Kit (Qiagen). DNA from both CB-HSCs and differentiated cells were treated with sodium bisulfite using EpiTect Bisulfite Kit (Qiagen) according to the related protocol. DNA samples treated with Sss1 enzyme and bisulfate, and untreated samples were used as positive and negative controls, respectively.

Methylation specific PCR

Methylation specific PCR was performed to evaluate the methylation status of the genes using bisulfite treated DNA of undifferentiated and miR-451 mediated differentiated cells. MSP reaction was conducted for the three genes using EpiTect MSP kit (Qiagen) according to the manufacturer´s procedure. The characteristics of primers and MSP reactions used in this step are listed in Table II.

Results

Purification and analysis of CD34$^+$ stem cells

In order to purify the cord blood stem cells CD34 surface marker was used as a specific marker for hematopoietic stem cells. The analysis of the flow cytometry results revealed that approximately 80% of cord blood cells were purified for CD34 after the MACS based purification procedure (Figure 1). We further selected this population for methylation analysis and mRNA expression of target genes as well as for differentiation purpose.

The enhancement of Erythroid specific markers in miR-451 mediated differentiated cells

In order to confirm erythroid differentiation after miR-451 treatment, both flow cytometry and RT-PCR method

were applied. The flow cytometery results demonstrated 65% expression for CD71 surface marker compared with isotype control (Figure 2, left). To confirm the flow cytometry results, the cDNA samples specific for CD71 and CD235 surface markers were resolved through 1% agarose gel electrophoresis. As it is indicated in Figure 2 (right) there is a significant difference between the expression levels of CD71 and CD235 specific cDNAs before and after differentiation. The high expression of CD71 and CD235 genes in differentiated cells well confirmed the miR-451 efficacy for erythroid differentiation. Evaluation of the CD71 mRNA level accompanied by flow cytometry analysis to measure the surface expression of the corresponding protein demonstrated that the marker was not only over-expressed in the mRNA levels after miR-451 treatment but also this enhancement is in the protein level as well.

The significant upregration of VHL, ECAD, and RUNX3 genes after erythroid differentiation

Relative quantification of the target genes *VHL, ECAD*, and *RUNX3* was performed by qRT-PCR before and after differentiation. The results showed that the miR-451 treated cells were highly enriched in the target genes compared with CB-HSCs. Figure 3 (A) shows the results for the expression levels of target genes before and after miR-451 mediated erythroid differentiation. The expression levels of VHL, ECAD, and RUNX3 genes in differentiated cells were increased 10.5, 112 and 37 folds, respectively, in comparison with CB-HSCs. The expression levels were normalized using the expression levels of GAPDH in each sample. In order to check the efficiency of miR-451 transfection, the levels of miR-451 was analyzed using miRNA qPCR before and after treatment. RNA extractd from differentiated cells proved to be highly enriched in miR-451 with levels of expression being 85%. Normalized values for miR-451 expression levels are indicated in Figures 3 (B). Inconsequential amplification of scramble sequence confirmed the accuracy of miR-451 expression results.

The partial methylation of ECAD and *RUNX3* genes in HSCs and differentiated cells

DNA extracted from CB-HSCs and miR451 mediated differentiated cells after bisufite treatment produced partial amplification with methylated and unmethylated primers for *ECAD* and *RUNX3* genes. Sharp bands for positive control and very weak or invisible amplification for negative control DNA suggests complete bisulfate conversion for unmethylated genes, so it does not provide a false positive results or interfere with the ability to distinguish methylated from unmethylated patterns. No significant difference between ECAD and RUNX3 methylated and unmethylated patterns in CB-HSCs and differentiated cells points out this fact that there is partial methylation of these two genes before and after erythroid differentiation (Figure 4, 5).

The strong unmethylation of VHL gene before and after differentiation by miR-451

MSP results provided invisible amplification for VHL gene in CB-HSCs and differentiated cells with methylated primer, (Figure 4, 5). Highly amplified pattern with unmethylated specific primers for VHL promoter suggests an unmethylated status for the region of promoter.

TableI: Characterization of primers in real time PCR

Oligo name	**5′ to 3′ Sequence**
VHL-F	CTGCCCGTATGGCTCAACTT
VHL-R	GTGTGTCCCTGCATCTCTGAAG
ECAD-F	GTCATCCAACGGGAATGCA
ECAD-R	TGATCGGTTACCGTGATCAAAA
RUNX3-F	GACAGCCCCAACTTCCTCT
RUNX3-R	CACAGTCACCACCGTACCAT

Table II: The primer characteristics for VHL, ECAD and RUNX3 genes used in MSP reaction

Gene	**Product size (bp)**	**Cycles**	**Annealing Temp (°C)**	**Form***	**5′ to 3′ Sequences**
VHL	165	35	60	UF	GTTGGAGGATTTTTTTGTGTATGT
				UR	CCCAAACCAAACACCACAAA
	158	35	60	MF	TGGAGGATTTTTTTGCGTACGC
				MR	GAACCGAACGCCGCGAA
ECAD	97	35	59	UF	TTAGTTAATTAGTGGTATGGGGGGTGG
				UR	ACCAAACAAAAACAAACACCAAATACA
	116	35	59	MF	TAATTAGCGGTACGGGGGGT
				MR	CGAAAACAAACGCCGAATACG
RUNX3	130	37	59	UF	GTGGGTGGTTGTTGGGTTAGTGAG
				UR	CACCTCCTCAACCACCACTACCAC
	130	37	59	MF	GTCGTCGGGTTAGCGAGGTTTCGT
				MR	CGACCGACGCGAACGCCTCCTC

* UF: Unmethylated Forward, UR: Unmethylated Reverse, MF: Methylated Forward, MR; Methylated reverse.

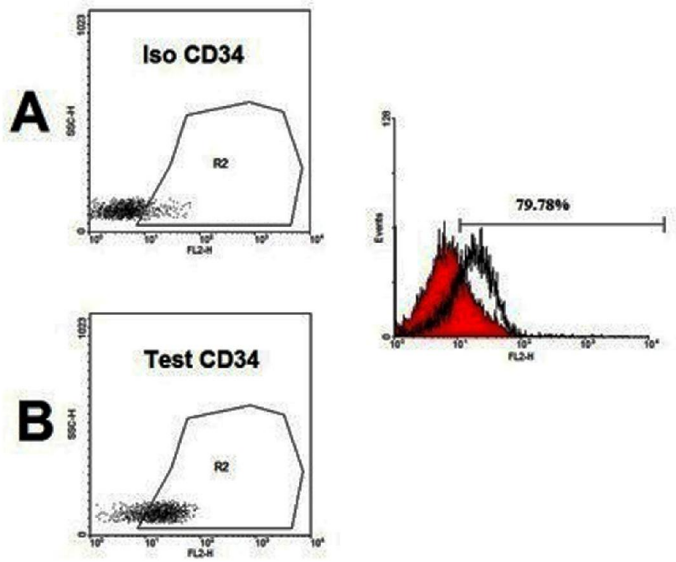

Figure1. Flow cytometry results of isolated human cord blood CD34+ cells labeled with CD34-PE. (A) Isolated CD34+ cells stained with isotype matched control labeled to PE. (B) Isolated CD34+ cells labeled to PE. The right Histogram showing approximately 80% purified CD34+ cord blood cells.

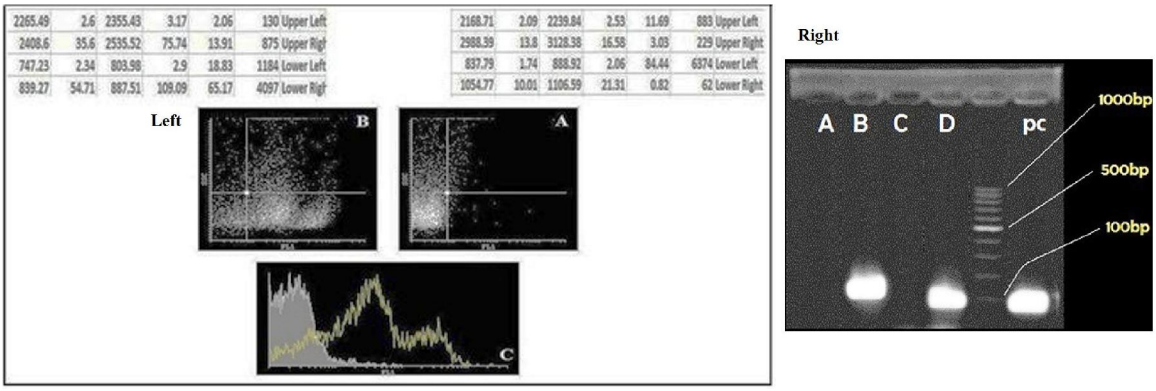

Figure 2. Assessment of differentiation efficiency (Left): Flow cytometry analysis regarding the expression of CD71 surface marker after erythroid differentiation by miR-451. (A) Staining of differentiated cells with isotype matched control labeled to FITC. (B) CD71 expression after miR-451 mediated erythroid differentiation in cells conjugated to FITC. (C) Histogram showing 65% differentiated cells regarding the expression of CD71 surface marker. (Right): Analysis of CD71 and CD235 gene expression after miR-451 mediated erythroid differentiation compared with undifferentiated cells. (A) And (B) represent the CD71 cDNA in undifferentiated and miR-451 mediated differentiated cells, respectively. (C) And (D) represent the CD235 cDNA in undifferentiated and miR-451 mediated differentiated cells, respectively. (pc: positive control using beta-actin as a housekeeping gene)

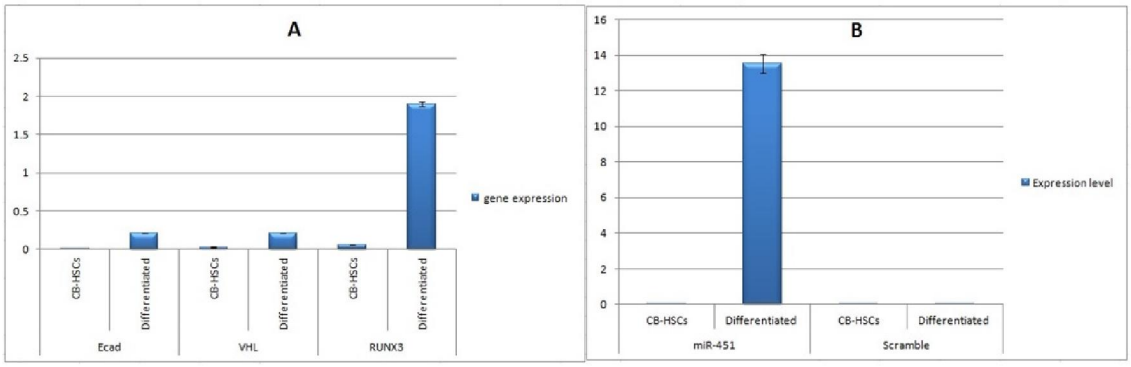

Figure 3. Quantification results for the target genes and miR-451. (A): Expression levels of ECAD, VHL, and RUNX3 genes before and after miR-451 mediated erythroid differentiation normalized to housekeeping GAPDH gene. As it is indicated all the target genes are significantly upregulated after miR-451 treatment. CB-HSCs, Cord Blood Hematopoietic Stem Cells (Before differentiation), bars labeled with "Differentiated" represent the gene expression levels after miR-451 mediated differentiation. (B): Expression levels of miR-451 in CB-HSCs and miR-451 treated cells (Differentiated) compared with Scramble expression status shows a highly increase in miR-451 detection after differentiation which well confirms the efficiency of miRNA transfection.

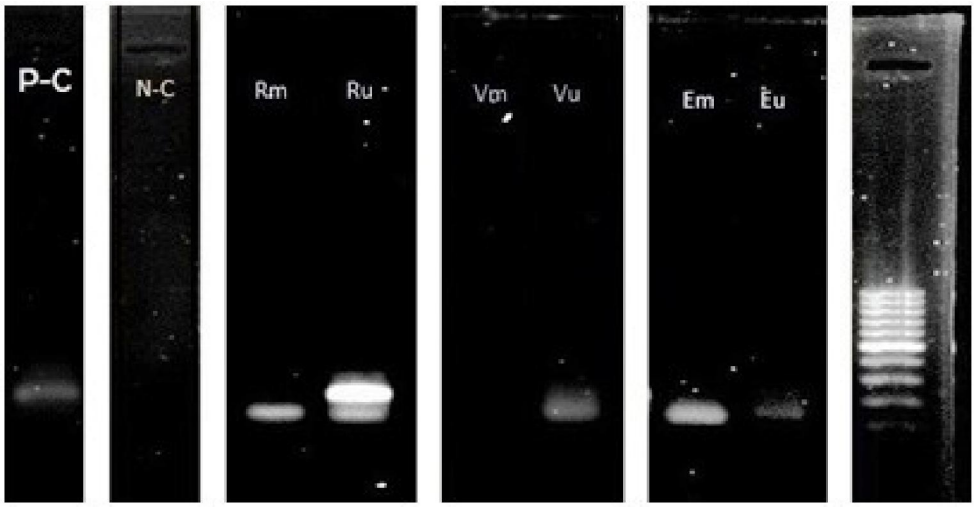

Figure 4. Methylation patterns for VHL, ECAD, and RUNX3 promoters before differentiation. Amplification patterns for RUNX3 and ECAD genes show a partial methylation status while for VHL genes indicate an unmethylated pattern. P-C and N-C are indicating positive and negative controls, respectively. M lines represent the reactions in which methylated forms of primers have been used, and U lines indicate the reactions in which the primers used in unmethylated forms. (R: RUNX3, V: VHL, E: ECAD).

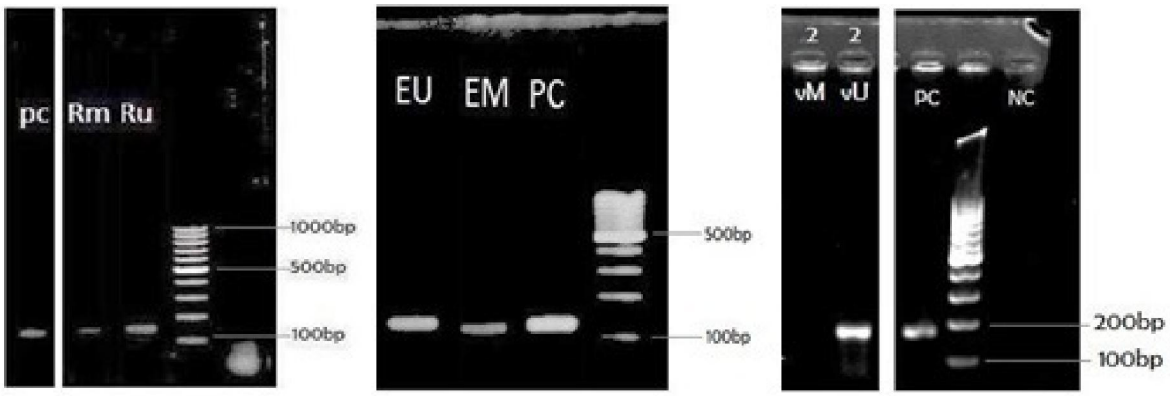

Figure 5. Methylation patterns for VHL, ECAD, and RUNX3 promoters after miR-451 mediated differentiation. Amplification patterns for RUNX3 (Left), ECAD (Middle), and VHL (Right) genes reveal similar methylation status as pre-differentiation stage (a partial methylation pattern for RUNX3 and ECAD, and an unmethylated pattern for VHL promoter). M lines represent the reactions in which methylated forms of primers have been used, and U lines indicate the reactions in which the primers used in unmethylated forms. (R: RUNX3, V: VHL, E: ECAD).

Discussion

A body of valuable concepts on tissue formation and gene regulation has been presented so far, but many of precise mechanisms through which stem cells differentiate into specific lineages have yet to be fully determined. Currently, it has been deciphered that stem cell commitment into specialized cell lineages is mainly controlled via gene expression level which in turn can be regulated by transcription factors, cell cycle regulators and signaling pathways (23-27). Present study brings together three seemingly separate processes which might contribute the differentiation of HSCs towards erythroid lineage. Furthermore, miRNA pathway was recently proved to regulate erythroid development (28, 29); moreover, the role of transcription factors and cell cycle regulators is also strongly evidenced (30, 31). In this study, we sought to characterize the interconnection among these three pathways by which CB-HSCs get restricted to erythroid lineage. For this purpose, we differentiated the CD34+ CB-HSCs into erythroid lineage by miR-451 and then, the expression levels and methylation patterns of VHL, ECAD and RUNX3 genes as contributing factors to erythroid differentiation have been compared before and after differentiation. Having the CpG-rich regions in their promoter these genes have potential to be controlled by DNA methylation, but our results contradicted this assumption. It is valuable to note that MSP method is not able to disclose subtle differences in methylation quantity, but it is possible that there may be little changes in DNA methylation which has not been detected via this method. More sensitive methods such as bisulfate sequencing may be able to clarify the methylation pattern of these genes more precisely. Moreover, it can be assumed that other probable mechanisms such as loss of heterogeneity, protein miss-localization or histone modification, may be involved in controlling these genes during erythroid differentiation which are required to come under careful scrutiny. Up-regulation of all the three genes especially ECAD in post differentiation stage confirms the important role of these factors in erythroid development. Moreover, basal expression of these genes in undifferentiated cells is in consistent with the" multi lineage gene priming" theory during which several lineage-specific genes of erythroid, myeloid, and lymphoid are transcribed at low level in HSCs and early progenitor cells.

Strong expression of VHL, ECAD and RUNX3 genes in miR-451 mediated differentiated cells could introduce these genes as potential targets for miR-451 during erythroid differentiation. It is possible that miR-451 may upregulate these genes by suppressing the specific inhibitors of the genes during erythroid differentiation which needed to be more scrutinized. Our results clearly indicated the parallel upregulation of VHL, ECAD, and RUNX3 after differentiation. But whether upregulation of these three genes is attributed directly to miR-451effects or caused by indirect mechanism is yet the subject to be fully enlightened. Owing to unchanged methylation pattern, it is supposed that methylation is not responsible to cause alteration in expression level of these genes.

MiR-451 was utilized to induce in vitro differentiation of CB-HSCs into erythroid lineage. These cells were differentiated successfully suggesting the efficacy of miR-451 for erythroid differentiation. Strong expression of CD71 surface marker detected on differentiated cells substantiated the effectiveness of miR-451 not only in upregulation of CD71 gene (as a determinant of erythroid lineage) in transcriptional level, but also in translational stage of CD71 mRNA. However, additional works with high population are needed to clarify the genetic and biologic basis of these malignancies.

Conclusion

Conclusively, significant upregulation of VHL, ECAD, and RUNX3 genes in differentiated erythroid lineage compared with CB-HSCs along with unchanged methylation pattern propose that control of these genes may not be associated with DNA methylation during erythroid differentiation and other mechanisms might be involved in this process. Another assumption is that expression level of a particular gene can be affected by the methylation level; therefore some genes may be regulated by small changes in methylation status which cannot be detected via MSP method. Further comprehensive studies are needed to fully determine the precise interaction between different mechanisms controlling the gene regulation and miR-451 expression during erythroid differentiation. Shedding light into these complicated mechanisms may pave the way to design targeted therapeutic measurements to alter the diseases such as anemia, cancer and other possible hematological disorders. More studies are need to focused on other microRNA and their proteins to elucidate the exact mechanisms controlling the gene expression during erythroid differentiation and also the crosstalk between these mechanisms.

Conflict of interest

The authors declare that they have no conflict of interest.

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